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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ReGen Therapeutics Plc

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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

A British Company

7672132001

4. Title of the invention

PEPTIDES

5. Name of your agent *(if you have one)*

A. A. THORNTON & CO.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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75001

Patents ADP number *(if you know it)*

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Continuation sheets of this form

Description 12

Claim(s)

Abstract

Drawing(s)

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Priority documents N/A

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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)* 1

Request for preliminary examination and search *(Patents Form 9/77)*

Request for substantive examination *(Patents Form 10/77)*

Any other documents *(please specify)*

11. I/We request the grant of a patent on the basis of this application.

Signature A.A. Thornton & Co. Date 2nd June 1999

12. Name and daytime telephone number of person to contact in the United Kingdom Philip A. Curtis - 0171-405 4044

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PEPTIDES

The present invention relates to peptides. More particularly the invention relates to certain peptides isolated from colostrinin. The invention also relates to therapeutic uses of the peptides and to antibodies derived therefrom.

Colostrum is the thick, yellowish fluid produced by a mammalian mother's breasts during the first few days after childbirth. It is the first lacteal secretion post parturition and it contains a high concentration of immunoglobulins (IgG, IgM and IgA) and nonspecific proteins. It is replaced by mature breast milk about four to five days after birth. Compared with mature breast milk, colostrum contains low sugar and iron. However, colostrum is richer in lipids, proteins, mineral salts, vitamins and immunoglobulins. It also contains various floating cells such as granular and stromal cells, neutrophils, monocyte/macrophages and lymphocytes and includes growth factors, hormones and cytokines.

Various factors have been isolated and characterised from mammalian colostrum. In 1974, Janusz et al (FEBS Lett., 49, 276-279) isolated a proline-rich polypeptide (PRP) from ovine colostrum. It has since been discovered that mammals other than sheep have analogues of PRP as a component of their colostrum. PRP has since been called colostrinin (and is sometimes called colostrinine) and has been tentatively identified as a new class of cytokine.

M. Janusz & J. Lisowski in "Proline-Rich Polypeptide (PRP) - an Immunomodulatory Peptide from Ovine Colostrum" (Archivum Immunologiae et Therapiae Experimentalis, 1993, 41, 275-279) mentioned that PRP from ovine colostrum has immunotropic activity in mice.

A. Dubowska-Inglot et al in "Colostrinine: a proline-rich polypeptide from ovine colostrum is a modest cytokine inducer in human leukocytes" (Archivum Immunologiae et Therapiae Experimentalis, 1996, 44, 215-224) discussed the use of colostrinin in the treatment of Alzheimer's disease.The use of colostrinin in the treatment of Alzheimer's disease, and other conditions, was also discussed in WO-A-98/14473.

Colostrinin, in its natural form, is obtained from mammalian colostrum. As described in WO-A-98/14473, analysis by electrophoresis and chromatography has

shown that colostrinin is a polypeptide having the following properties:

(i) it has a molecular weight in the range 16,000 to 26,000 Daltons (this was shown by electrophoresis in the presence of SDS);

(ii) it is a dimer or trimer of sub-units each sub-unit having a molecular weight in the range 5,000 to 10,000 Daltons (this was shown by acrylamide gel electrophoresis in the presence of SDS);

(iii) it contains proline, and the amount of proline is greater than the amount of any other single amino acid (this can be shown by conventional amino acid analysis).

By means of these techniques it was shown that ovine colostrinin has a molecular weight of about 18,000 Daltons, is made up of three non-covalently linked sub-units each having a molecular weight of about 6,000 Daltons and includes about 22 wt% proline. The amino-acid composition of ovine colostrinin was shown to be made up of the following number of residues per sub-unit: lysine - 2, histidine - 1, arginine - 0, aspartic acid - 2, threonine - 4, serine - 3, glutamic acid - 6, proline - 11, glycine - 2, alanine - 0, valine - 5, methionine - 2, isoleucine - 2, leucine - 6, tyrosine - 1, phenylalanine - 3 and cysteine - 0.

We have now further analysed the composition of colostrinin in order to try to identify its components, so that a synthetic form of colostrinin can be produced.

We have concluded that colostrinin contains N-terminal amino acid sequences from at least two different proteins: annexin; and β- casein. In addition, colostrinin contains a number of other N-terminal amino acid sequences which do not have any known precursor protein; these amino acid sequences may be derived from an unknown precursor protein, or they may have no precursor protein (for example, they may be a collection of new cytokines).

According to one aspect of the present invention there is provided a peptide of having one of the following amino acid sequences 1-31:

1. MQPPPLP
2. LQTPQPLLQVMMEPQGD
3. DQPPDVEKPDLQPFQVQS
4. LFFFLPVVNVLP

5. DLEMPVLPVEPFPFV
6. MPQNFYKLPQM
7. VLEMKFPPPPQETVT
8. LKPFPKLKVEVFPEP
9. VVMEV
10. SEQP
11. DKE
12. FPPPK
13. DSQPPV
14. DPPPPQG
15. SEEMP
16. KYKLQPE
17. VLPPNVG
18. LNE
19. VYPFTGPIPN (Position 74-83)
20. SLPQNILPL (Position 84-92)
21. TQTPVVVPPF (Position 93-102)
22. LQPEIMGVPKVKEMVPK (Position 103-120)
23. HKEMPFPKYPVEPFTESQ (Position 121-138)
24. SLTLDVEKLHLPLPLVQ (Position 139-156)
25. SWMHQPP (Position 157-163)
26. QPLPPTVMFP (Position 164-173)
27. PQSVLS (Position 174-179)
28. LSQPKVLPVPQKAVPQRDMPIQ (Position 180-201)
29. AFLLYQEPVLGPV (Position 202-214)
30. RGPFPILV (Position 214-222)
31. ATFNRYQDDHGEEILKSL (Position 203-220)

We also found that colostrinin contains the following peptide, which is disclosed in WO-A-97/14473:

32. VESYVPLFP

These peptides may be provided in substantially isolated form. Furthermore, a

composition may be provided which contains two or more of the above peptides, in combination.

The invention also includes any peptide or peptide fragment which includes an N-terminal amino acid sequence which is the same as that in peptides 1-32. Thus, with reference to peptide 1, for example, the invention encompasses any peptide having the N-terminal amino acid sequence MQPPPLP; the same applies to peptides 2-32.

The peptides 1-18 and 32 did not have any known precursor protein. The peptides 19 to 20 were derived from β-casein (the position on the β-casein amino acid chain being stated in brackets after each peptide). The peptide 31 was derived from annexin (the position on the annexin amino acid chain being stated in brackets after the peptide).

The peptides can be obtained by a number of techniques. In one embodiment, they can be prepared by isolation from colostrinin. In a preferred embodiment, they are prepared by a conventional synthetic technique, such as by solid-phase peptide synthesis.

The peptides, either alone or in combination with one another, have a number of therapeutic uses.

In one advantageous embodiment, one or more of peptides 1-32 may be used in the treatment of disorders of the central nervous system, particularly chronic disorders of the central nervous system. The disorders of the central nervous system that may be treated include neurological disorders and mental disorders. Examples of neurological disorders that may, with advantage, be treated include dementia, and also disorders that cause dementia, such as neurodegenerative disorders. Neurodegenerative disorders include, for example, senile dementia and motor neurone disease; Parkinson's disease is an example of a motor neurone disease that can be treated. Alzheimer's disease is an example of a neurodegenerative disease that can be treated. Examples of mental disorders that can be treated by one or more of the peptides include psychosis and neurosis. For example, the peptides may be used to treat emotional disturbances, especially the emotional disturbances of psychiatric patients in a state of depression. The peptides may also be used as an auxiliary withdrawal treatment for drug addicts, after a period of detoxification, and in persons

dependent on stimulants.

In another advantageous embodiment of the invention, one or more of peptides 1-32 may be used in the treatment of disorders of the immune system, particularly chronic disorders of the immune system the may occur spontaneously in people of advanced age. The peptides can also be used in the treatment of disease requiring immuno-modulation. The peptides are useful in the treatment of a variety of diseases with an immunological and infectious basis. For example, they can be used to treat chronic diseases with a bacterial and viral aetiology, and to treat acquired immunological deficiencies that have developed, for example, after chemotherapy or radiotherapy of neoplasms. The peptides may be used for treating chronic bacterial and viral infections requiring non-specific immunostimulation and immunocorrection.

A chronic disorder is a disorder that has persisted, or is expected to persist, for a long time, i.e., at least 3 months and usually at least 6 months.

One or more of the peptides may be used for improving the development of the immune system of a new born child. It is a further feature of the invention to use the peptides to correct immunological deficiencies in a child. These uses of the peptides may be particularly applicable to babies or children who have been deprived of colostrum. This may occur, for example, in babies and children who were not breast fed from birth.

The peptides, either alone or in combination with one another, also have diagnostic and research applications. For example, the synthetic peptides, as well as the corresponding antibodies described below, may be used to recognise pathological processes occurring in a host. These processes may be induced by excessive production or inhibition of the peptides or the antibodies. Once the pathological process associated with a particular level of the peptides or the antibodies is known, measuring the production of the peptides and the antibodies in body fluids may be used to determine pathological processes taking place in the host.

According to another aspect of the invention, we provide the use of one or more of peptides 1-32 as a dietary supplement. This dietary supplement is particularly useful for babies, especially premature babies and babies at term, and for young children to correct deficiencies in the development of their immune system. The dietary

supplement may also be used as a dietary supplement for adults, including senile persons, who have been subjected to chemotherapy, or have suffered from cahexia, or weight loss due to chronic disease.

In an aspect of the invention, we provide a dietary supplement comprising an orally ingestible combination of one or more of peptides 1-32 in combination with a physiologically acceptable carrier. The dietary supplement may be provided in liquid or solid form; the dietary supplement may suitably be provided in the form of a tablet. The dietary supplement may be provided in the form of a baby food formula. The dietary supplement may include, as an additive, lactoferrin and/or selenium and/or a group of cytokines containing members of the interferon family.

In accordance with the invention, one or more of peptides 1-32 may be administered prophylactically in order to help to prevent the development of disorders of the central nervous system and the immune system.

The peptides according to the invention may be administered in a dosage in the range 1 ng to 10 mg. A dosage unit of about 3 µg is typical.

The peptides according to the invention may be formulated for administration in any suitable form. For example, they may be formulated for oral, rectal or parenteral administration. More specifically, the peptides may be formulated for administration by injection, or, preferably, in a form suitable for absorption through the mucosa of the oral/nasopharyngeal cavity, from the alimentary canal or any other mucosal surface. The oral formulations may be provided in a form for swallowing; or, preferably, in a form for dissolving in the saliva, whereby the formulation can be absorbed in the mucous membranes of the oral/nasopharyngeal cavity. The oral formulations may be in the form of a tablet for oral administration, lozenges (i.e. a sweet-like tablet in a form suitable to be retained in the mouth and sucked), adhesive gels for rubbing into the gum. The peptides may be formulated as an adhesive plaster or patch, which may be applied to the gums. The peptides may also be formulated for application to mucous-membranes of the genito-urinary organs.

One or more of the peptides may be incorporated into products like milk or cheese spread.

We have found that the ratio of the peptides in colostrum varies over time.

Owing to hormonal changes, many proteins secreted into colostrum become sequentially degraded. The longer the time from parturition the more extensive the degradation can be. This knowledge will help with the design of new baby food formulas as well as many drugs for immuno-compromised patients.

In another aspect, the invention provides the antibody for each of the peptides 1-32, and provides compositions containing said antibodies. The antibody for each peptide can be produced in rabbits, for example, by injecting the peptide with adjuvant. This technique is described in detail in Example 3. It is possible to test that the correct antibody has been produced by ELISA (enzyme-linked immunosorbent assay) using the synthetic peptides as antigens. The antibodies can be further tested against the natural peptides in colostrinin as confirmation that the synthetic peptides do correspond to the natural peptides found in colostrinin. The antibodies have potential uses in therapy, as a diagnostic tool and as a research tool.

The invention also encompasses the selective administration of one or more of peptides 1-32, at selected times to the patient, and the selective administration of one or more of the antibodies for the peptides in order to switch on or off the activity of the peptides at a selected time.

A selection of selected ones of the peptides and/or antibodies may be provided in a single composition which is specially tailored to produce a particular effect. For example, for a person with an immunological disorder, the composition can be specially tailored for that disorder. The composition may be specially selected for more than one disorder. The composition may be specially selected to restore or produce a particular balance in a subject.

The invention will now be further described with reference to the following examples.

Example 1

Preparation of Colostrinin

Colostrinin can be prepared by techniques already disclosed in the prior art, including, for example, WO-A-98/14473. Colostrum collected from the ewe within 12 hours post parturition can be purified by centrifuging to eliminate cellular and lipidic

components, pH shifting to eliminate nutritional components, ammonium sulfate precipitation, ion exchange chromatography and molecular sieving.

Example 2

Identification of the Components of Colostrinin

The colostrinin produced in example 1 was fractionated using hplc (high performance liquid chromatography) using a C-18 reverse-phase column. This technique was used to separate the peptides, exhibiting different hydrophobic patterns, present in colostrinin. The hplc column was obtained from Separation Methods Technologies (who are based in Newark, Delaware, U.S.A). The column type was designated C18 and was 150 mm in length by 10 mm in diameter. The particle size was 3 µm having a pore size of 30 nm. The pump module and diode array were supplied by Beckman (who are based in Fullerton, California, U.S.A.): a Beckman System Gold 126 pump module was used, and a Beckman System Gold 168 diode array detector module was used. The flowrate in the column was 0.06 ml/min, and the solvent composition was varied as shown in Table 1.

Table 1

Time/Min	% Solvent A	% Solvent B
0.00	95.0	5.0
10.00	30.0	70.0
100.00	0.0	100.0
140.00	95.0	5.0
150.00	95.9	5.0

Solvent A: 0.1% TFA (trifluoroacetic acid) in hplc grade water.

Solvent B: 70% acetonitrile fluoride and 0.09% TFA in hplc grade water.

The peptides found at the peaks in the hplc were then individually analysed using Edman Degradation; this was done using a Beckman LF3000. Each concentrated

fraction was loaded into a pre-salted Beckman peptide support disk. The samples were sequenced using the standard Edman degradation steps. Subsequently, each fraction was analysed by the Inline hplc System. This used a Hewlett Packard PTH-AA column having a length of 250 mm and a diameter of 2.1 mm. The Beckman System Gold 126 pump module was used, and the Beckman System Gold 168 diode array detector module was used. The flowrate in the column was 0.275 ml/min, and the solvent composition was varied as shown in Table 2.

Table 2

Time/Min	% Solvent A	% Solvent B
0.00	80.0	20.0
0.10	62.0	38.0
17.10	10.0	90.0
28.10	87.5	12.5

Solvent A: 3.5% THF (tetrahydrofuran), 1.5% acetonitrile fluoride premix, 1% acetic acid & 0.02% TEA (triethanolamine) in hlpc grade water.

Solvent B: 12% isopropanol in acetonitrite.

By means of these techniques we were able to identify all of peptides 1 to 32.

Example 3

Formation of the Antibodies

The peptides identified in example 2 were produced by synthetic techniques. The a cysteine group was then added to the N-terminal end of each synthetic peptide, and the peptide was formed into a ring so that the cysteine group lay between the N-terminal and the C-terminal ends of the synthetic peptide. These synthetic peptides rings were then used as antigens to produce antibodies in rabbits using a protocol well known in the art. The protocol used followed the following sequence:

Day	Procedure

	0	Prebleed & initial inoculation of rabbit with 200 µg of the peptide at 0.5 ml of conjugate solution mixed with an equal volume of complete Freund's adjuvant (mineral oil/emulsifier/killed mycobacteria).
5	14	Boost inoculation with 200 µg of the peptide at 0.5 ml of conjugate solution mixed with an equal volume of incomplete Freund's adjuvant (mineral oil/emulsifier).
	28	Boost (as on day 14)
		Production Bleed (approx. 20ml sera)
10	42	Boost (as on day 14)
		Production Bleed (approx. 20ml sera)
	56	Boost (as on day 14)
		Production Bleed (approx. 20ml sera)
	70	Boost (as on day 14)
15		Production Bleed (approx. 20ml sera)

This protocol may be varied. For example, the frequency of the production bleed depends upon, inter alia, the size and health of the host species.

The sera produced by the bleeds were subjected to ELISA (enzyme-linked 20 immunosorbent assay) with the corresponding peptide antigen. This technique involved the following steps:

1. The antigen was diluted with a bicarbonate buffer (pH 9.0) to yield a 10 µg/ml solution. A volume of 50 µl of this solution was placed into each microwell of a 96 well plate.

25 2. The plates were covered and incubated at 37°C for 3 hours.

3. The wells were blocked using 200 µl of blocking BSA (bovine serum albumin).

4. 50 µl of dilutent BSA (0.75% soln.) was pipetted into each well. 50 µl of antibody serum sample diluted 1:100 in dilutent BSA were placed in lane 30 A of each row.

5. 1:2 serial dilutions were performed moving down the plate.

6. The plates were covered and incubated at room temperature for 60 minutes.

7. The plates were washed four times with PBS (phosphate-buffered saline) wash solution.

8. A volume of 50 µl of goat anti-rabbit IgG (H&L) HRP conjugate at 1:1000 dilution in BSA was pipetted into each well and incubated at room temperature for 60 minutes (H&L = heavy and light chain; HRP = horseradish peroxidase).

9. The plates were washed four times with PBS wash solution.

10. A volume of 50 µl of substrate solution (ABTS, containing 2,2 azo dye,3-ethylbenzothiazoline sulphide - this dye is used to help visualise the extent of the antibody/antigen reaction) was pipetted into each well and incubated at room temperature for about 2 minutes.

11. The reaction was stopped by adding 50 µl of 2% SDS (sodium dodecyl sulfate) into each well.

12. The wells were then read on a dynoplate reader at 405.

The titer results of the ELISA for Peptides 1-8 are shown in Table 3. Table 3 also indicates the OD_{280} and IgG values for each antibody.

Table 3

Antibody for:	Titer	OD_{280}	IgG mg/ml
Peptide 1	1:12800	9.634	6.88
Peptide 2	1:12800	8.733	6.24
Peptide 3	1:25600	9.653	6.9
Peptide 4	1:25600	6.948	4.96
Peptide 5	1:25600	7.090	5.06
Peptide 6	1:25600	6.000	4.29
Peptide 7	1:25600	10.150	7.25
Peptide 8	1:25600	10.066	7.19

("OD" = Optical Density)

These results indicate that the potency of the antibodies produced in respect of peptides 1-8 was excellent, and therefore that each antibody was the correct antibody for its synthetic peptide antigen.

The antibodies produced by this technique were monospecific.

Example 4

In order to show that peptides corresponding to the synthetic peptide antigens exist in colostrinin it was necessary to show that the antibodies produce a reaction in colostrinin itself.

We studied the rate at which the peptides 1 to 8 disappeared from colostrum produced in sheep. The colostrum was collected from the mother's milk at 24 hours, 48 hours and 72 hours post parturition, and the level of peptides 1 to 8 was measured. The peptide level was measured by means of an antigen-antibody reaction, using the antibodies produced by the method of Example 3.

Table 4

Peptide:	24 Hours	48 Hours	72 Hours
1	12800	12800	3200
2	6400	3200	3200
3	12800	12800	6400
4	12800	3200	3200
5	12800	6400	3200
6	6400	3200	3200
7	6400	6400	3200
8	12800	6400	3200

These results demonstrated that the amino acid sequences 1-8 had been correctly decoded and that the antibodies made from the synthetic peptides recognise the peptides contained in colostrinin.

It will be appreciated that the invention described above may be modified.